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Endogenous brain-sparing responses in brain pH and PO_2 in a rodent model of birth asphyxia

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Abstract

Aim: To study brain-sparing physiological responses in a rodent model of birth asphyxia which reproduces the asphyxia-defining systemic hypoxia and hypercapnia.

Methods: Steady or intermittent asphyxia was induced for 15-45 minutes in anaesthetized 6- and 11-days old rats and neonatal guinea pigs using gases containing 5% or 9% O_2 plus 20% CO_2 (in N_2). Hypoxia and hypercapnia were induced with low O_2 and high CO_2 respectively. Oxygen partial pressure (PO_2) and pH were measured with microsensors within the brain and subcutaneous ("body") tissue. Blood lactate was measured after asphyxia.

Results: Brain and body PO_2 fell to apparent zero with little recovery during 5% O_2 asphyxia and 5% or 9% O_2 hypoxia, and increased more than twofold during 20% CO_2 hypercapnia. Unlike body PO_2, brain PO_2 recovered rapidly to control after a transient fall (rat), or was slightly higher than control (guinea pig) during 9% O_2 asphyxia. Asphyxia (5% O_2) induced a respiratory acidosis paralleled by a progressive metabolic (lact)acidosis that was much smaller within than outside the brain. Hypoxia (5% O_2) produced a brain-confined alkalosis. Hypercapnia outlasting asphyxia suppressed pH recovery and prolonged the post-asphyxia PO_2 overshoot. All pH changes were accompanied by consistent shifts in the blood-brain barrier potential.

Conclusion: Regardless of brain maturation stage, hypercapnia can restore brain PO_2 and protect the brain against metabolic acidosis despite compromised oxygen availability during asphyxia. This effect extends to the recovery phase if normocapnia is restored slowly, and it is absent during hypoxia, demonstrating that exposure to hypoxia does not mimic asphyxia.

KEYWORDS

brain pH and oxygen, brain protection, graded restoration of normocapnia, HIE, perinatal asphyxia, physiology

1 | Introduction

Severe birth asphyxia (BA; also known as perinatal asphyxia) is the main cause of disability and mortality of human neonates worldwide, with more than one million casualties annually.[1] The number of the surviving, afflicted individuals is not known, but there is reason to believe that it is much higher. Thus, BA makes a significant contribution to the total burden of disease in human populations, based on aberrant development and dysfunctions of organs, which are highly reliant on oxidative energy metabolism, especially the brain. The immediate pathological effect of BA on the brain manifests as hypoxic-ischaemic encephalopathy (HIE), and the lifelong outcomes of HIE include a wide spectrum of psychiatric and neurological diseases and disorders, including cognitive defects, autism, epilepsy and cerebral palsy.[2-6]

Therapeutic hypothermia is currently the only generally accepted treatment for near-term and term newborns with moderate or severe BA, but it provides incomplete neuroprotection.[7-11] In addition to HIE, BA causes a wide spectrum of (often causally connected) dysfunctions including those of the adrenals and the heart. Obviously, advances in understanding the basic physiology and pathophysiology of BA and the mechanisms that lead to HIE and adverse lifelong outcomes will promote development of more effective therapies.[12]

By definition, BA implies a decrease in systemic O_2 (hypoxia) and an increase in CO_2 (hypercapnia). A period of asphyxia occurs also during normal uncomplicated deliveries, and this plays an essential role in triggering endogenous mechanisms that operate to centralize blood flow to critically oxygen-dependent organs.[13-15] Augmenting endogenous neuroprotective mechanisms or supplementing their effectors has been frequently suggested as basis for novel therapeutic interventions for BA.[16,17]

Regarding the translational value of animal models, understanding of the systems-physiological mechanisms involved in BA and related conditions[18] has been significantly improved by elegant work on pathophysiological and intrinsic protective mechanisms in large-animal models such as sheep and pigs.[14-16] However, these mechanisms have remained largely unexplored in standard laboratory rodents. Extending the systems-level work on BA from large animal models to laboratory rodents will offer the potential of studying the mechanistic aspects of BA and its consequences using the vast array of neurobiological research methods available today, from molecules to systems, which have been largely tailored for rats and mice.

Our present experiments are mainly based on postnatal day (P) 6 and 11 rat pups which, in terms of neurodevelopmental (especially cortical) milestones, roughly correspond to preterm and full-term human neonates respectively.[19-22] Asphyxia is induced by applying an ambient gas mixture containing 5% O_2 and 20% CO_2 (balanced with N_2) using two paradigms: *monophasic asphyxia* ("steady asphyxia") which corresponds to an acute complication such as placental abruption or maintained cord compression, and *intermittent asphyxia* where the hypoxia is applied in repetitive steps at 5% O_2 and 9% O_2 (at constant 20% CO_2) in order to roughly mimic the effects of recurring contractions during prolonged parturition. We use here infant rats of two ages, because even if a given insult would have similar immediate effects, the long-term outcomes in eg brain functions and behaviour (not studied presently) will depend on the stage of development of the brain at the time of insult.[19,23] We also included experiments on P0-2 guinea pigs to provide a more comprehensive translational basis for this study, and also because there is a recent increase in experimental work on early-life disorders in this species.[24,25]

The main aim of our present work is to examine the complex and interconnected effects of BA on brain pH and oxygen partial pressure (PO_2) levels. These are the two fundamental physiological variables which are strongly affected at the onset, during and after asphyxia. Notably, systemic acidosis (typically blood pH <7.0) is used as one of the standard diagnostic criteria of BA. Both pH and PO_2 are known to be powerful modulators of the function of practically all organs and organ systems, including cardiorespiratory regulation[13,26] and the excitability of the immature brain.[27,28] Regarding the latter, a large variety of key molecules involved in neuronal signalling show a functionally synergistic (most likely evolutionary-contingent)[29] sensitivity to pH, whereby brain acidosis acts to suppress neuronal excitability while alkalosis has the opposite effect.[29-31] This notion gets further impact in the context of BA from observations that experimental hypoxia as such (ie not as a component of asphyxia) leads to a gross increase in neuronal network excitability as has been shown in both *in vivo* and *in vitro* conditions.[32-36] "Pure" hypoxia, ie hypoxia without simultaneous hypercapnia – which never takes place in natural conditions – has dominated thinking not only in translational research of hypoxic-ischaemic brain damage (see Discussion) but also in clinical practice, particularly regarding methods of resuscitation.[37] The differences between pure hypoxia and asphyxia are further underscored by the profound influence of CO_2 on cerebral blood flow (CBF).[13] Thus, understanding the physiological and pathophysiological consequences of BA, including the propensity for subsequent manifestation of HIE, requires information on (a) the magnitude and time course of perturbations of acid-base regulation and oxygenation in brain tissue, and on (b) the adaptive mechanisms which act in a "brain-sparing" protective manner.

Using pH- and O_2-selective microsensors implanted into the brain and subcutaneous tissue ("body"), we show here that experimental BA produces a fast and large CO_2-mediated (respiratory) acidosis, which is combined to a slow metabolic acidosis that is much smaller within than outside brain tissue.

A striking action by brain-sparing mechanisms was observed in simultaneous measurements of brain parenchyma and body PO_2, which demonstrated a full restoration of the brain PO_2 in response to steady and intermittent exposure to 9% O_2 in the presence of 20% CO_2.

This adaptive mechanism was absent under conditions of pure 9% hypoxia. Moreover, combined to the unexpected brain *alkalosis* which we observed immediately in response to pure hypoxia, these data show that exposure of an infant rodent to hypoxia[32,38] does not reproduce the physiological responses to asphyxia. Finally, we demonstrated that Graded Restoration of Normocapnia (GRN) following asphyxia, a putative therapeutic strategy,[39] slows down the pro-excitatory recovery of brain pH and extends the duration of the post-asphyxia PO_2 overshoot. Thus, in the neonatal intensive care unit, judicious tailoring of the GRN protocol would provide a possibility to block the frequent and deleterious post-BA hypocapnia.[40,41] By slowing down the restoration of normocapnia, it should also be possible to set the duration of the post-asphyxia PO_2 overshoot to enhance brain oxygenation immediately after birth.[42,43]

2 | Results

2.1 | Changes in brain and body pH induced by asphyxia, hypercarbia and hypoxia in P6 rats

Birth asphyxia is associated with systemic acidosis that has a respiratory and a metabolic component because of CO_2 accumulation and to O_2 deficit respectively. We first carried out a series of experiments on P6 rats in order to analyse changes in extracellular brain pH and in subcutaneous tissue pH (brain pH and body pH, respectively; see Materials and Methods). Recording of body pH using pH-sensitive microelectrodes is continuous and therefore avoids problems associated with repeated blood sampling and it provides a continuous measure of systemic pH with good correlation with blood pH.[44] Below, we also addressed separately the effects of hypoxia and hypercapnia, the two components of asphyxia, on brain and body pH.

2.1.1 | Steady asphyxia

The mean brain pH at baseline in all experiments at P6 was 7.31 ± 0.01 (mean ± SEM, n = 32). Asphyxia (5% O_2 and 20 CO_2; for 45 min) caused immediately a rapid fall in brain pH, followed by a very slow and progressive acidification with a smaller amplitude (Figure 1A). The fall in pH within the first 10 minutes was 0.47 ± 0.01 (n = 6) with a maximum fall rate of 0.21 ± 0.02 pH units per min, whereas the subsequent slow acidification was 0.10 ± 0.02 with a rate of 0.003 ± 0.0005 pH units per min, reaching a final pH of 6.75 ± 0.02 by the end of the 45 minutes asphyxia period.

The post-asphyxia recovery of brain acidosis was very fast (Figure 1A). It consisted of two phases, where the first 15 minutes was a mirror image of the asphyxia-induced rapid fall in pH, and it was followed by a second phase after about 20 min. Compared to brain pH changes seen previously upon a more moderate asphyxia (9% O_2 and 20% CO_2),[39] the present results were different in that (a) brain pH did not start to recover during asphyxia but decreased, albeit slowly, until the end of the asphyxia period, and (b) the final pH level at the end of the recovery period was only slightly higher than the initial baseline pH (by 0.05 ± 0.02, $P = .034$; paired *t*-test).

The mean body pH at baseline in all experiments at P6 was 7.34 ± 0.01 (n = 32). In contrast with the brain pH response during asphyxia, body pH changes did not have two kinetically distinct phases. Although initially slower than in the brain (maximum rates of acidosis in body and brain mean pH were 0.051 and 0.21 pH units per minute, respectively), the fall in body pH reached a higher amplitude of 0.70 ± 0.02 pH units at 45 minutes (n = 6; $P = .00007$, paired *t*-test; pH 6.65 ± 0.02). Also, the recovery of body pH was slower. The temporal behaviour of the difference (ΔpH = brain pH – body pH) is illustrated in the middle panel in Figure 1A. Notably, a slow shift in the alkaline direction of brain vs body pH develops during asphyxia, which does not fully fade out by the end of the 90 minutes recovery.

2.1.2 | Hypercapnia

Hypercapnia induced by 20% CO_2 caused a prompt fall in brain pH with a maximum rate (0.21 ± 0.03 pH units per min) and amplitude (0.45 ± 0.01 at 10 min; n = 6; $P = .294$; Figure 1B) similar to what was seen in asphyxia. However, in contrast with asphyxia, there was no further slow acidosis but, instead, a small but consistent rise of 0.03 pH units after the peak acidosis of 6.85 ± 0.01 at 17.6 ± 1.5 minutes of hypercapnia (final pH at 45 minutes 6.89 ± 0.02). In contrast with asphyxia, hypercapnia led to a fall in body pH which attained a steady maximum amplitude that was almost identical to that generated simultaneously in the brain (0.48 ± 0.02, n = 6 vs 0.43 ± 0.02 at 45 min, respectively; $P = .099$, paired *t*-test; final body pH at 45 minutes 6.84 ± 0.04). Notably, such pH changes are very similar to the "passive" (ie purely physicochemical) acid shift of 0.6 pH units that would be generated by an increase in CO_2 from 5% to 20% in a physiological CO_2/bicarbonate solution with no other buffers.[45] This suggests that the rapid fall in brain pH, which constitutes

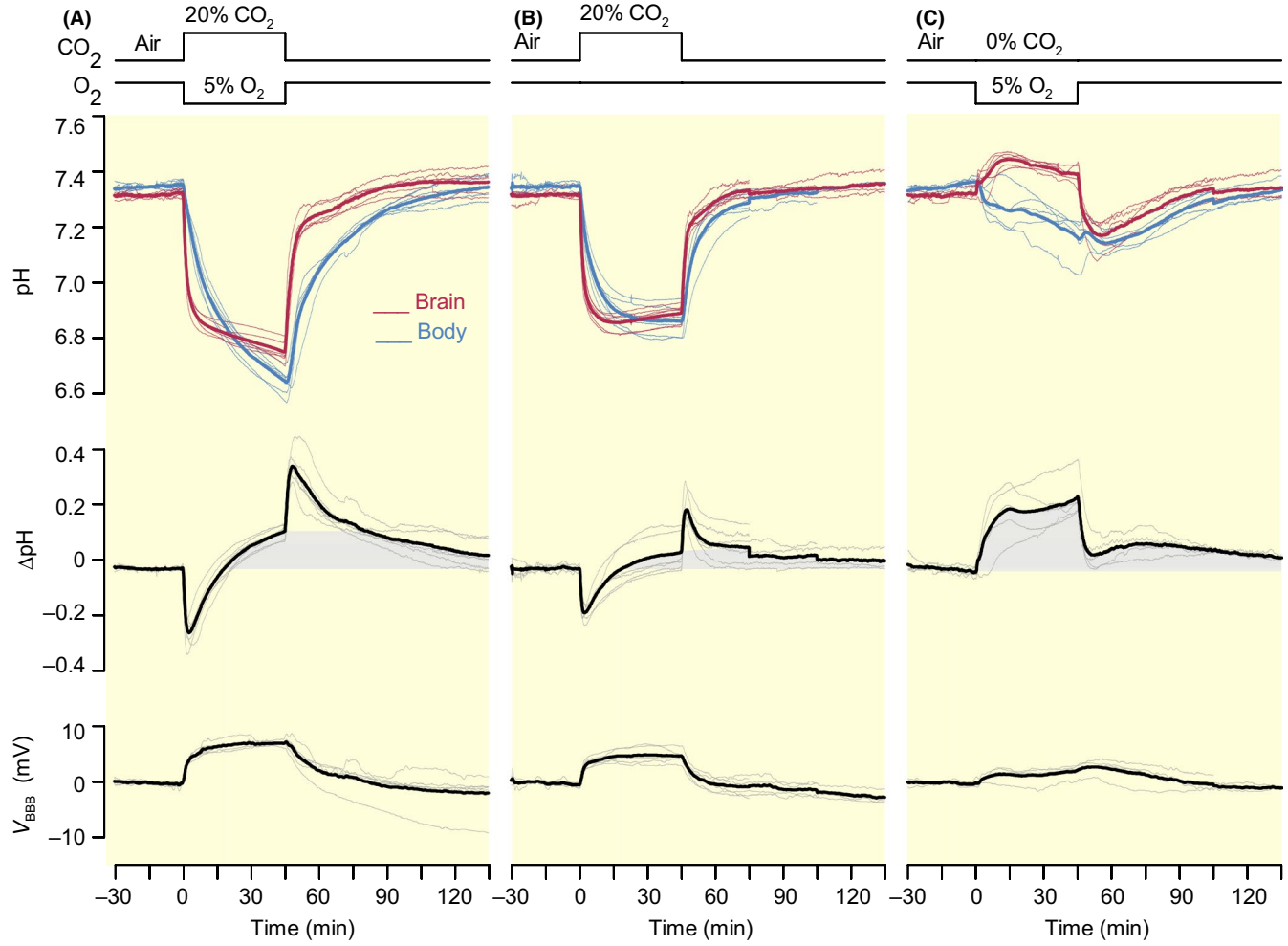

FIGURE 1 pH responses induced by experimental asphyxia, hypercapnia and hypoxia in P6 rats. (A) Brain (red) and body (blue) pH responses to 45 min 5% O_2 asphyxia (top panel), their difference (middle panel) and the blood-brain barrier potential V_{BBB} (bottom panel). In this and subsequent Figures, superimposed thin and thick traces show individual recordings and their mean respectively. The line graphs above data traces indicate timing with which the animals were exposed to different gases. Grey shading is used to highlight the difference of brain and body pH, excluding the transient shifts caused by the slower respiratory body pH response upon changes in inhaled CO_2. (B) Responses to 45 min hypercapnia induced by 20% CO_2. (C) Responses to 45 hypoxia induced by reducing the O_2 content in inhaled gas to 5%. In this and subsequent Figures some traces of individual recordings do not continue until the end of the recovery phase because recording was discontinued, which accounts for the small stepwise shifts seen in the mean traces

a major part of brain acidosis during experimental asphyxia, is primarily caused by the increase in CO_2 in the brain extracellular space where the non-CO_2 buffer power is low.[46] The faster responses in brain vs body pH (see the transient "on" and "off" responses in the ΔpH traces in Figure 1) are readily accounted for by more effective perfusion of brain tissue compared to the subcutaneous site of body pH measurement.

Thus, striking and physiologically relevant differences were observed when comparing the neonates' responses in body pH to asphyxia and hypercapnia. In particular, the above data indicate that the experimental asphyxia triggered a metabolic acidosis that is much larger in the body than in the brain.

While consistent with metabolic acidosis during the experimental asphyxia, the above dynamic pH data provide indirect evidence for its generation. Therefore, we took blood samples from P6 rats and, indeed, found that the present asphyxia protocol elevates blood lactate in only 15 minutes from 0.88 ± 0.15 mmol L^{-1} (n = 5) in controls to 11.1 ± 0.80 mmol L^{-1} (n = 5), see also ref. [47]. This result is of further (translational) importance given that generation of lactic acid is largely responsible for the base deficit (negative base excess) which is one of the key diagnostic criteria of BA.

2.1.3 | Hypoxia

As a whole, the above data suggest that an endogenous mechanism protects the brain against metabolic acidosis and base deficit during asphyxia. This idea gained further

support from the simultaneously measured brain and body pH responses to hypoxia (5% O_2; Figure 1C). Unexpectedly, and in sharp contrast with what was seen during asphyxia, brain pH measurements demonstrated an *alkaline* shift upon hypoxia. Brain pH reached a maximum increase of 0.13 at 16 ± 1.5 minutes (7.44 ± 0.011 vs baseline, n = 5; $P = .0003$, paired *t*-test) and, while losing amplitude, it remained above control throughout the 45 minutes hypoxia period (in 5 of 5 recordings). Return to air caused a transient rebound acidosis followed by recovery of brain pH to the control level. The alkaline pH response suggests that hypoxia triggers net extrusion of acid equivalents across the blood-brain barrier (BBB) at a rate that exceeds net acid production within the brain parenchyma.

The effective compartmentalization at the level of the BBB was also seen in measurements of body pH during hypoxia, in which a gradual monophasic acidification (amplitude at 45 minutes 0.18 ± 0.04, n = 5) with no initial alkalosis was seen. This is reminiscent of the dynamics of the slow (apparently metabolic, see above) body acidosis during asphyxia. Together, these responses resulted in a robust positive shift in ΔpH lasting throughout the hypoxia (Figure 1C). A comparable prolonged positive shift in ΔpH was evoked also by asphyxia but not by hypercapnia (see the shaded areas in Figure 1 that indicate the slowly-generated positive (brain more alkaline) ΔpH and where the ΔpH transients caused by step changes in inhaled CO_2 have been excluded).

As a final conclusion based on the data in Figure 1, the time courses and amplitudes of brain and body pH changes related to asphyxia (Figure 1A) seem to behave roughly like sums of the pH responses triggered by the two underlying components, hypercapnia (Figure 1B) and hypoxia (Figure 1C), recorded in isolation.

2.2 | pH-induced changes in the trans-BBB potential in P6 rats

The BBB maintains a pH-sensitive potential difference between brain tissue and the rest of the body (V_{BBB}).[48,49] We monitored the V_{BBB} signal by measuring changes in the voltage between the brain and body (see Materials and Methods). The acid shifts induced by asphyxia or hypercapnia were tightly paralleled, as expected, by positive shifts in V_{BBB} with maximum amplitudes of 7.1 ± 0.18 mV (n = 6) and 4.9 ± 0.49 mV (n = 6) respectively ($P = .0015$). The response in V_{BBB} upon hypoxia was positive which, together with its smaller amplitude and time course, suggests a dependence on body pH, not on brain pH. Previous work shows that respiration-induced mV-level slow EEG shifts that are generated by the human BBB can be readily measured non-invasively using DC-coupled EEG.[49] Thus, measuring DC-EEG shifts may open up a new window for brain monitoring during recovery from BA (see Discussion).

2.3 | Brain and body PO_2 changes upon asphyxia, hypercapnia and hypoxia in P6 rats

The results above raise questions about oxygen availability and consumption in brain vs body during the experimental manoeuvres. As CO_2 is a well-known vasodilator,[13] we next carried out tissue PO_2 recordings in P6 rats.

The mean baseline levels of brain and body PO_2 based in all experiments on the P6 rats were 20.8 ± 0.9 mmHg, n = 54, and 26.0 ± 1.8 mmHg, n = 23 respectively. These levels are much lower than (a) PO_2 in inhaled air, which is about 160 mmHg, and (b) arterial blood PO_2 of 90 to 100 mmHg, which corresponds to normal 96% to 98% oxygen saturation,[50] consistent with tissue PO_2 levels reflecting a balance between O_2 delivery and consumption.

The responses in brain and body PO_2 to asphyxia, hypercapnia and hypoxia are illustrated and described in detail in Supporting Information. The main findings were as follows: Asphyxia (5% O_2/ 20% CO_2) made both brain and body PO_2 fall instantaneously to apparent zero (see Materials and Methods), and their recovery after asphyxia occurred with a large transient overshoot. During hypercapnia (20% CO_2) brain and body PO_2 increased 3.6- and 2.7-fold respectively. Not surprisingly at all, brain PO_2 was at apparent zero during pure 5% O_2 hypoxia, and even the more moderate hypoxia with 9% O_2 made both brain and body PO_2 rapidly fall and stay at near zero levels. Our data indicate that brain consumes all available O_2 during 5% O_2 asphyxia and during 5% to 9% O_2 hypoxia but, as such, a near-zero O_2 level within brain parenchyma does not justify the conclusion that brain energy metabolism is primarily anaerobic under these conditions. Furthermore, our results raise the question: How much O_2 is needed to avoid severe tissue hypoxia when the brain-sparing mechanisms are active during asphyxia? To address these issues and to advance the translational potential of the experiments, we next recorded both pH and PO_2 responses with the intermittent asphyxia protocol.

2.4 | Intermittent asphyxia reveals an enhancing effect of elevated CO_2 on brain oxygenation in P6 and P11 rats

In order to gain a deeper insight into the dependence of pH and PO_2 on the developmental stage and severity of asphyxia, we used an experimental paradigm to mimic the intermittent O_2 supply that is typical to BA.[26] Here, we used both P6 and P11 pups which roughly correspond to preterm and full term babies in the developmental stage of the cerebral cortex.[19,51]

In P6 rats, 9% / 5% O_2 intermittent asphyxia (see scheme in Figure 2; and Materials and Methods) caused an acid shift and recovery in both brain and body pH with characteristics very similar to those seen in steady 5% O_2 asphyxia, except for the smaller amplitude of acidosis in both compartments (brain 6.81 ± 0.01 and body 6.76 ± 0.014, n = 8, at the end of asphyxia; Figure 2A). In line with this, the response in trans-BBB potential differed from steady asphyxia mainly by its somewhat smaller amplitude. The alternation between 5% and 9% O_2 in inhaled gas mixture gave rise to relatively small shifts (< 0.035) in both pH signals. The intermittent asphyxia is expected to induce a smaller metabolic acidosis than the steady one and this is, indeed, evident in the brain and body pH traces which diverge much less in the former (compare the shaded areas under ΔpH traces in Figures 1A and 2A).

An interesting observation on PO_2 responses, particularly those in the brain, was made with intermittent asphyxia (n = 8 and 4 for brain and body, respectively; Figure 2A). At the beginning of asphyxia, in 9% O_2/20% CO_2, the two PO_2 signals fell rapidly but started to recover within a minute indicating the activation of some compensatory mechanism(s). During the subsequent 5 minutes period with 5% O_2, both brain and body PO_2 fell to zero, in a manner similar to under conditions of 5% O_2 asphyxia. When the O_2 was then increased from 5% to 9% for 5 min, brain PO_2 started to rapidly recover towards its control level, despite the continuous hypoxic level of ambient O_2. During the subsequent three steps to the 9% level, the rise in brain PO_2 became even larger, reaching—and in 5 of the 8 animals crossing—the pre-asphyxia control level during the last 9% O_2 period (Figure 2A). Notably, the parallel partial

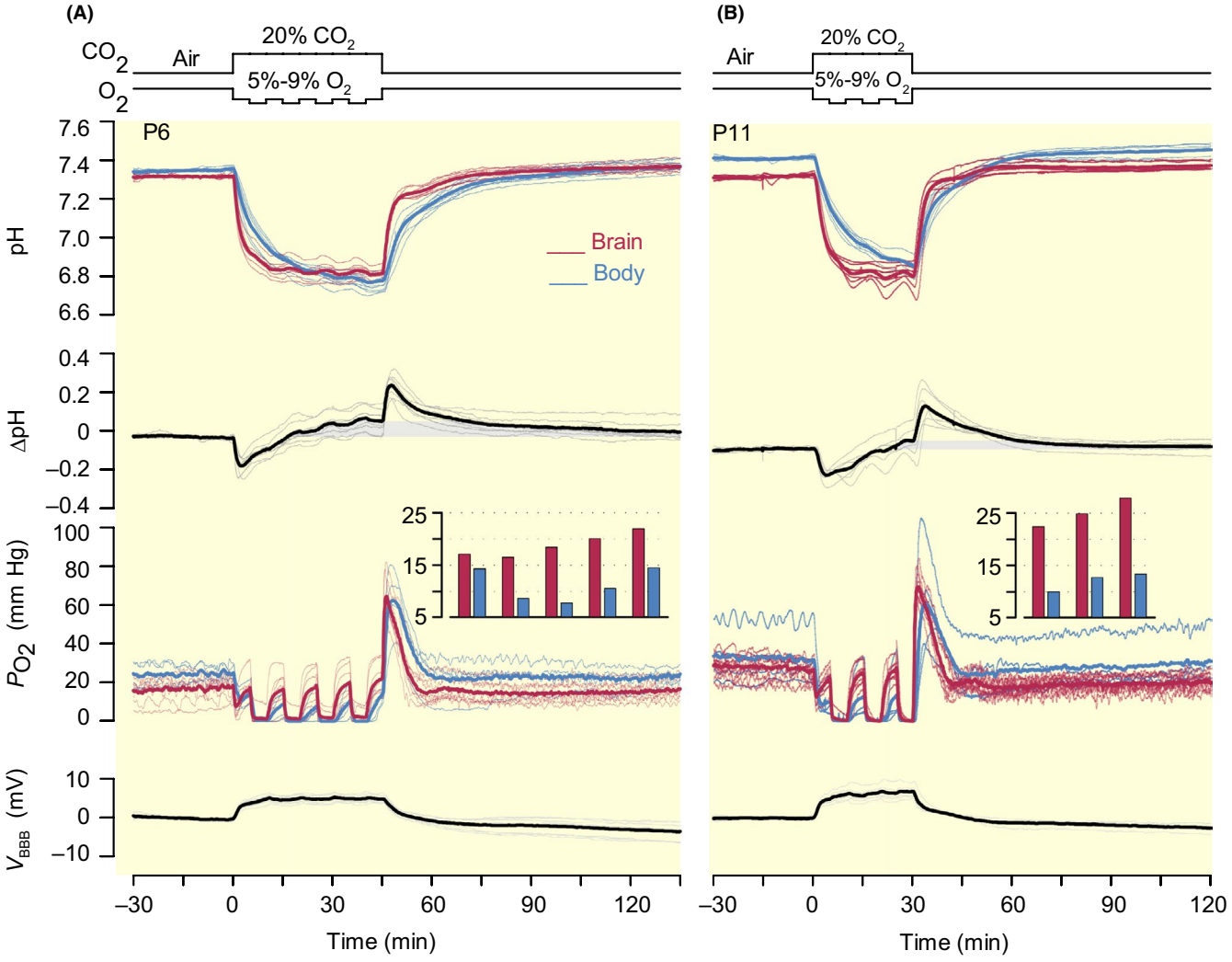

FIGURE 2 pH and PO_2 responses induced by intermittent asphyxia in P6 and P11 rats. (A) Simultaneously recorded responses to 45 min intermittent asphyxia in brain and body pH as well as in V_{BBB} are shown in parallel with brain and body PO_2 responses obtained in a separate series of otherwise identical experiments. All data in (A) are from P6 rats. (B) Similar to (A) but with a 30 min intermittent asphyxia applied on P11 rats. The bar graph insets in (A) and (B) show the maximum values of mean brain and body PO_2 that were reached by the end of each 5 min period of inhaling 9% O_2, 20% CO_2

recovery of body PO_2 was much smaller, with its final peak at around half of the control level, indicating that the compensatory mechanism[15] postulated above works in a more efficient manner in the brain.

Return to air evoked a transient overshoot in both brain and body PO_2, very similar to those seen after 5% O_2 steady asphyxia. Comparing to the steady asphyxia, the less severe nature of the intermittent asphyxia likely accounts for the lack of the body PO_2 undershoot below its baseline level during the time interval of approximately 30-60 minutes after return to breathing air.

The above results clearly show that even a limited source of O_2 during asphyxia can be sufficient for restoring normoxic conditions in the brain at P6. During development, increasing energy metabolism paralleled by angiogenesis[52,53] is expected to have consequences on brain pH and PO_2 responses. Therefore, we next used the intermittent asphyxia model with P11 rats and, based on pilot experiments, we decreased the duration of exposure to 30 minutes to keep mortality at zero. The mean baseline brain pH of all P11 rats (7.31 ± 0.01, n = 11) did not differ from that at P6 ($P = .91$), but baseline body pH was slightly higher (7.41 ± 0.02, n = 11; $P = .0043$). The mean baseline brain PO_2 in the P11 rats (31.7 ± 0.91 mmHg, n = 32), was noticeably higher than in P6 rats ($P = 5 \bullet 10^{-12}$). A comparable increase at P11 was seen in the body PO_2 (34.2 ± 2.06 mmHg, n = 22; $P = .0043$). As is evident from Figure 2B, there were no obvious differences in the responses evoked by intermittent asphyxia at P11 compared to those at P6. In a series of experiments with hypercapnia like the one in Figure S1B but at P11 (not illustrated), the increases in PO_2 upon any of the three CO_2 levels (5%, 10%, 20%) differed neither in the brain nor in the body from those at P6 (P values from 0.38 to 0.94, n values from 4 to 10). Like in P6 rats, brain and body PO_2 fell rapidly to zero during 9 % O_2 hypoxia in P11 rats (15 minutes exposure; n = 6 and 4 for brain and body, respectively; not illustrated), and no obvious differences were seen in the PO_2 responses during recovery compared to P6.

2.5 | Neonatal guinea pigs maintain higher PO_2 during severe asphyxia than rats

The guinea pig is a rodent which has been used in a number of translational studies on BA and other early-life disorders.[54] This species is adapted to life at high altitudes[55,56] and it has precocial neonates, which are born with regard to their cerebrocortical development at a stage that is much more advanced in comparison to the altricial neonatal rat and the full-term human newborn.[25] Thus, to study further the developmental and inter-species aspects of brain PO_2 responses, we used P6 and P11 rats as well as guinea pigs at P0–2 and compared their brain and body PO_2 responses when exposed to steady 5% or 9% O_2 asphyxia for 15 minutes.

As expected, 5% O_2 asphyxia resulted in both P6 and P11 rats in a prompt fall in brain PO_2 to apparent zero level, with little further change during the 15 minutes asphyxia (n = 10 and 7, respectively; Figure 3A top and middle panels). The recovery consisted in both age groups a transient rise that peaked at a level that was approximately twice higher than the baseline PO_2 value, followed by a slower fall to baseline level in about 15 min. The simultaneous body PO_2 responses resembled those in the brain but had somewhat slower kinetics and lower recovery overshoot (n = 4 for both P6 and P11 rats).

The moderate 9% O_2 steady asphyxia for 15 minutes showed again recovery with the typical transient overshoot in P6 and P11 rats, but a small difference was observed between brain and body PO_2 levels during the asphyxia period (Figure 3B top and middle panels). At P6, a very brief drop was followed by a rise in brain PO_2 to a level that was at or even above the baseline whereas the body PO_2 signal recovered from the initial drop more slowly and levelled off below its baseline (n = 6 and 5, respectively), ie brain and body were slightly hyperoxic and hypoxic, respectively, during the 9% O_2 steady asphyxia. At P11, the initial drop in both brain and body PO_2 was larger and only brain PO_2 showed a partial recovery during asphyxia, reaching $76.7 \pm 6.5\%$ of its baseline value, whereas body PO_2 levelled off at $27.1 \pm 10.7\%$ of its baseline level (n = 4 for both; Figure 3B middle panel).

In P0-2 guinea pigs, the baseline brain and body PO_2 were 31.6 ± 1.6 mmHg (n = 5) and 43.2 ± 6.8 mmHg (n = 3) respectively. In a manner similar to P6 rats, guinea pig brain tissue did not become hypoxic during the 9% O_2 asphyxia except for the very first <1 minutes, but instead rapidly increased to a level that was 9.2 ± 2.0 mmHg above baseline at 5 min, and then slowly declined towards the baseline level before a very brief overshoot to 82.9 ± 2.3 mmHg was evoked by return to breathing air (n = 5; Figure 3B bottom panel). The similarity to P6 rats was seen also in the response of guinea pig body PO_2 to 9% O_2 asphyxia (by the end of asphyxia, body PO_2 fell to 33.1 ± 0.7 mmHg, n = 3, ie to $81.3 \pm 15.4\%$ of baseline). Interestingly, during the severe 5% O_2 asphyxia, guinea pigs differed from both P6 and P11 rats in that their brain PO_2 fell rapidly to a transient minimum of $35.4 \pm 5.4\%$ of the pre-asphyxia baseline and then increased and stayed at no less than $61.0 \pm 9.9\%$ of baseline (16.9 ± 1.9 mmHg) till the end of asphyxia (n = 5; Figure 3A bottom panel). The simultaneously recorded body PO_2 showed a more robust, progressive fall that reached 8.7 ± 3.2 mmHg, ie $22.0 \pm 8.1\%$ (n = 3) of the pre-asphyxia baseline, by the end of the 15 minutes 5% O_2 asphyxia, followed by a moderate overshoot during recovery.

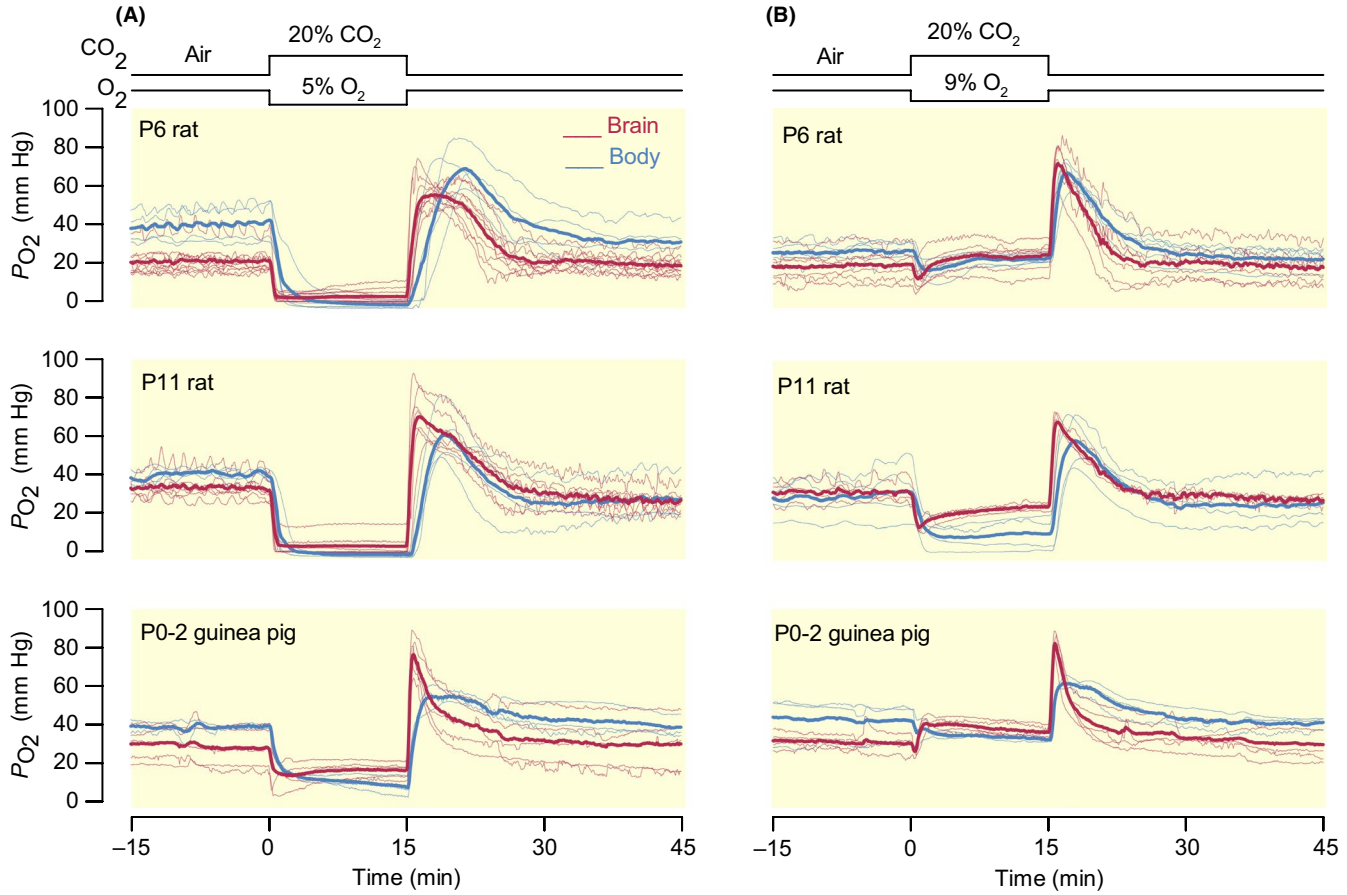

FIGURE 3 Brain and body PO_2 responses to severe and moderate asphyxia in rats at P6 and P11 as well as in P0-2 guinea pigs. (A) Top, middle and bottom panels show superimposed individual and mean traces of brain and body PO_2 recorded in P6 and P11 rats and P0-2 guinea pigs exposed to 5% O_2 asphyxia for 15 min. (B) Similar to (A) but with a more moderate, 9% O_2 asphyxia

2.6 | Effects of GRN on brain and body pH, and on brain PO_2, during recovery from asphyxia in P6 and P11 rats

GRN applied during recovery from asphyxia holds promise as a therapeutic intervention that protects the brain and improves the outcome.[39] Therefore, in an extensive series of experiments we focused on steady and intermittent asphyxia with GRN.

In contrast with the fast pH recovery seen in P6 rats during rapid restoration of normocapnia (RRN) after 45 minutes steady 5% O_2 asphyxia (Figure 1A), the recovery that was seen during GRN had three distinct phases in both brain and body (Figure 4A). As expected, these phases in pH recovery paralleled those in the ambient CO_2 levels, and therefore the recovery of both brain and body pH to baseline was much slower than with RRN. During GRN, body pH remained below brain pH (see ΔpH in Figure 4A), and the final pH levels at the end of recovery were identical with those seen with RRN ($P = .62$ and $P = .76$ respectively). Again, the V_{BBB} signal closely followed the time course of pH changes. Sudden V_{BBB} collapses were not associated with any of the experimental insults used in this study, which suggests that the insults did not cause BBB disruption.

In parallel experiments at P6, the transient post-asphyxia overshoot in brain PO_2 peaked rapidly at a level (77.9 ± 5.7 mmHg, n = 4) that was nearly three times higher than the baseline, and higher and broader than the peak seen during RRN (cf. Figure S1A; $P = .31$ for peak height comparison). Thereafter, PO_2 decreased slightly (to 63.6 ± 6.1 mmHg) by the end of the 30 minutes exposure to 10% CO_2 / 20% O_2 gas, followed by a further fall in brain PO_2 when CO_2 was lowered to 5%. The fall was slow and did not attain a stable level in 30 minutes (brain PO_2 60 minutes after end of asphyxia 32.5 ± 5.0 mmHg). Return to air 60 minutes after the end of asphyxia made brain PO_2 fall to a somewhat hypoxic level.

The effects of GRN with P11 rats after 30 minutes intermittent asphyxia (n = 4; Figure 4B) were consistent with those described above for P6 rats. The peak in brain PO_2 was higher and broader than with RRN at P11. Compared to GRN at P6, a stable brain PO_2 level was attained more rapidly during the 30 minutes recovery periods with 10% and 5% CO_2, and these levels were approximately 2 and 1.3 times higher than the mean baseline respectively. Return to

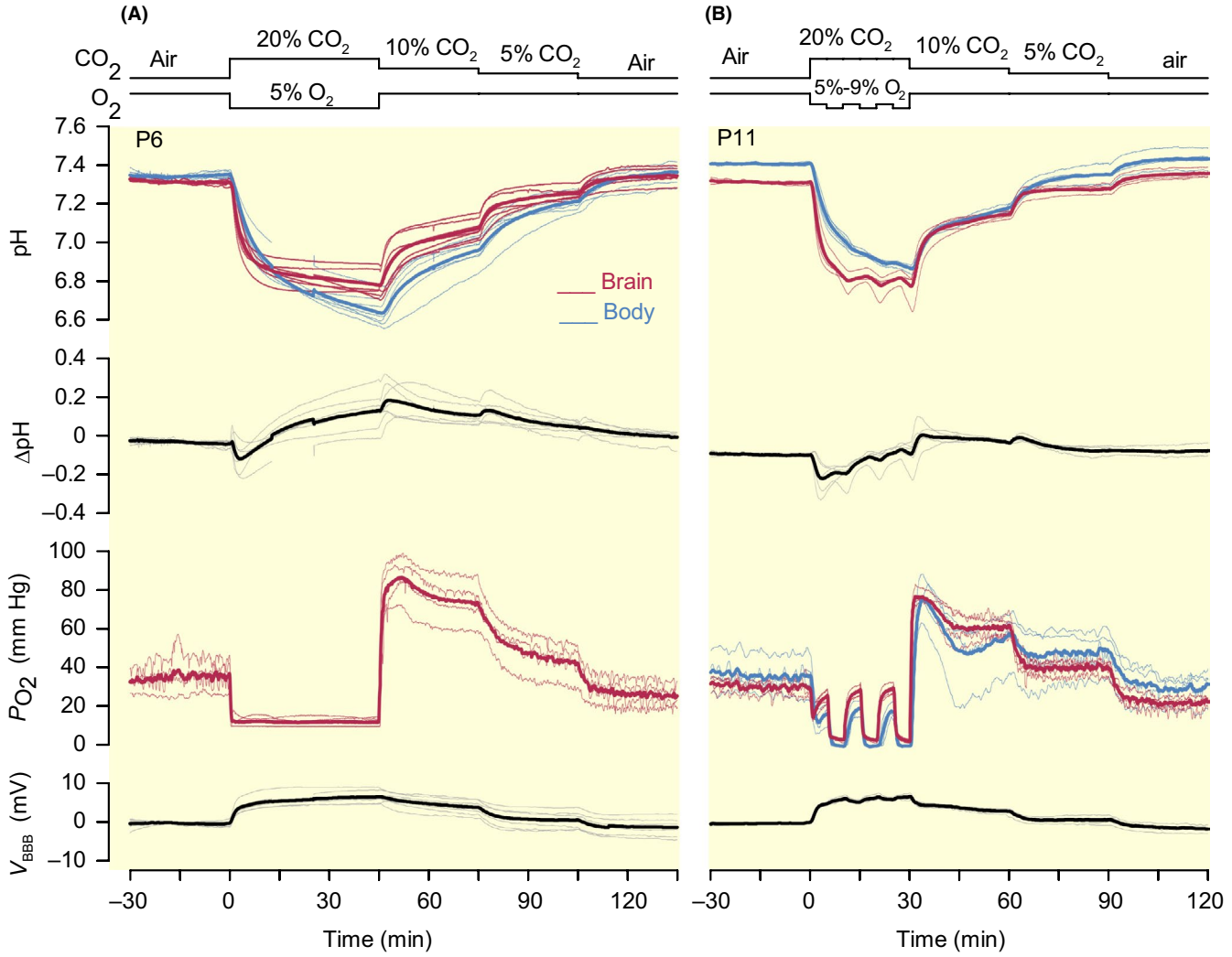

FIGURE 4 Graded restoration of normocapnia during recovery from asphyxia slows down the alkaline recovery and prolongs the PO_2 overshoot in P6 and P11 rats. (A) P6 rats were exposed to 5% asphyxia for 45 min after which recovery took place with graded restoration of normocapnia, ie normal ambient O_2 level was restored immediately whereas CO_2 in the inhaled gas was first decreased to 10% for 30 min followed by 5% CO_2 for another 30 min before the animals were finally breathing normal air. Superimposed individual and mean traces of simultaneously recorded brain and body pH and V_{BBB} are shown in parallel with brain PO_2 data from a separate series of otherwise identical experiments. (B) Similar to (A) but the experiments were done on P11 rats and using intermittent asphyxia for 30 min, and also body PO_2 was recorded simultaneously with brain PO_2. One of the body PO_2 traces deviates from corresponding traces recorded from other P11 rats and could therefore be considered as an outlier in data. However, since we were unable to identify any technical reasons for this atypical behaviour during early recovery, we did not discard this recording from the data analysis

air caused a fall in brain PO_2 to a hypoxic level (21.5 ± 1.3 mmHg) followed by a slow trend towards the original baseline value.

3 | DISCUSSION

In this study we have focused on pH and PO_2 as two key variables in the brain *milieu interior* in our rodent model of BA. We have examined the magnitude and time course of the perturbations caused by a number of experimental paradigms, including *severe* and *moderate asphyxia,* as well as severe and moderate *hypoxia* and *hypercapnia* in isolation (see Materials and Methods). This permitted the identification of adaptive mechanisms pointing to the remarkable ability of the immature rodent brain to cope with the low O_2 levels associated with asphyxia, as has been previously shown in extensive studies on larger mammals, such as sheep and pigs.[14-16] Moreover, our experiments indicate that these adaptive mechanisms are not at work during pure hypoxia, which is a condition that a mammal never faces—pre-, peri- or postnatally—under natural conditions. The present data will also increase our understanding on the conditions *in vivo* that will affect neuronal excitability during BA. Finally, we demonstrate that *slowing down restoration of normocapnia* after a period of asphyxia will prolong the actions of the

innate brain-protective mechanisms as well as those of hypercapnia itself.

Our main observations on pH are that (a) while the hypercapnia component of asphyxia is responsible for most of the large and fast acidosis seen in brain and body pH, the brain appears to be protected against the more slowly developing metabolic acidosis. We found that (b) unlike asphyxia, exposure to hypoxia produces a small but immediately triggered brain-confined *alkalosis* which is paralleled by a slow and similarly modest (by definition a metabolic) acidosis in the body, pointing to the BBB's capability of acid extrusion[57] at a rate that exceeds net acid production in the brain, as postulated earlier on,[58] see also ref. [59]. The hypoxia-induced net brain alkalosis shows that BA cannot be adequately mimicked by *in vivo* hypoxia (see below). Finally, (c) the fast rate of recovery of brain pH after asphyxia was significantly reduced by GRN, an effect that has a pronounced suppressing action on neocortical seizures as shown by Ala-Kurikka *et al.*[60] in a parallel study. Notably, in the present work done with anaesthesia and a slightly lower body temperature of 33.5—34 °C (see Materials and Methods) vs 36.5—37 C, no seizures took place.

With regard to oxygen levels, we found a striking difference if hypoxia was applied alone, or whether it was applied as one of the two components of asphyxia. During hypoxia (5% or 9% O_2) as well as during severe experimental asphyxia (ie 20% CO_2 plus 5% O_2), both brain and body PO_2 fell to levels that were close to zero. However, with 9% O_2 in parallel with 20% CO_2 hypercapnia (moderate asphyxia), the brain regained its control PO_2 level pointing to the activation of highly effective adaptive mechanisms. Moreover, our recordings show a prompt post-asphyxia overshoot of brain (and body) PO_2 which was prolonged by GRN.

The above effects as well as their mechanisms and consequences will be discussed in detail below.

3.1 | Towards a valid rodent model of birth asphyxia

3.1.1 | Characteristics of the present model

Choosing an experimental model to study BA is not a straightforward task. Obviously, *in vitro* models cannot reproduce systems level-adaptive responses to asphyxia. Because of the altricial nature of rat and mouse offspring, their cortical development corresponds around P6 to human preterm (from 28 up to 35 gestational weeks) and at around P11 to term babies.[19,21-22,51] Therefore, experimental manipulations must be designed in a manner that reproduces asphyxia-mimicking conditions in these postnatal animals, which have already acquired a fully functional pulmonary ventilation.

The main features of our model are the following: (a) It is noninvasive, and therefore the endogenous vasomotor and other systems-level[61,62] (eg neurohormonal)[24,47] protective mechanisms remain fully operational. (b) Compromised gas exchange via the umbilical cord, which results in both CO_2 accumulation and O_2 deficit in the foetus, is mimicked by elevating CO_2 and reducing O_2 in the ambient gas, instead of exposing the animal to a hypoxic gas which blocks the respiratory acidosis characteristic of asphyxia (see section on Asphyxia vs hypoxia below). (c) The experimental manipulations target the entire infant rat, with a tissue and organ distribution of adaptive and other physiological responses which are of endogenous origin. (d) Furthermore, our pH and lactate data indicate that the current model reproduces the key clinical criteria of severe BA in human neonates, namely systemic acidosis to pH levels <7.0 and a base deficit ≥12 mmol L^{-1}. [8,63,64] Regarding hypercapnia, umbilical cord artery gas partial pressure (Pa) values in neonates with severe acidosis at birth are typically >100 mmHg $PaCO_2$ (≥140 mmHg in >10% of cases), and PaO_2 is often in the range 10 to 15 mmHg.[65,66] Thus, in all the three versions of the present model (moderate, severe and intermittent asphyxia), CO_2 was maintained at the constant 20% level, while O_2 was reduced to 9% or 5% in order to unravel the animal's fully activated endogenous capacity to maintain brain oxygenation despite the limited oxygen resources.

The newborn guinea pig is precocial and therefore its brain at birth is much more mature than that of the altricial rat.[51-52,67] However, our results with P0 to P2 guinea pigs (which correspond to two to three week old rats in terms of cortical development)[25,52] were qualitatively identical to, and even quantitatively similar, to the results obtained with the P6 and P11 rats, suggesting that the present experimental approaches are valid in translational work on all standard laboratory rodents.

3.1.2 | Asphyxia vs hypoxia

Our present data on brain alkalosis induced by pure hypoxia speaks against the relevance of hypoxia models of asphyxia. Another important line of evidence comes from experiments on hypoxia on neuronal excitability. Despite a number of purinergic mechanisms activated during oxygen deprivation,[68,69] *in vivo* hypoxia alone is known to produce a gross increase in cortical excitability which becomes manifest as seizures *during* this challenge.[32-35] If, indeed, the hypoxia models are intended to mimic what happens in clinical BA, this would imply that the neonatal seizures associated with complicated birth are triggered already *in utero* and during parturition. This is obviously not the case. What is missing in hypoxia models of BA is hypercapnia, and the consequent respiratory acidosis that lowers brain excitability by modulating a wide variety of

voltage and ligand-gated ion channels, see ref. [29] and references cited therein. Thus, while hypoxia promotes neocortical excitability, hypercapnic acidosis has a functionally opposite effect. This is, notably, consistent with the fact that neonatal seizures caused by asphyxia take place with a substantial delay (several hours) after birth,[70] ie at a time when normoxic conditions have already been established. From a general neurobiological point of view, it is interesting to note that, under all natural conditions, hypoxia *in vivo* is associated with hypercapnia, suggesting an evolutionary history of the development of the neuromodulatory effects of pH *in vivo,*[29] whereby neuronal acidosis suppresses excitability.[71-73]

Thus, the recovery of brain pH is likely to be a factor that sets the time course of the increase in brain excitability during recovery from asphyxia. Worth mentioning is that in our previous study[39] we used isoflurane instead of urethane anaesthesia, and the initial depth of anaesthesia may have been too deep, likely accounting also the significantly (by >0.1 units) lower baseline brain pH compared to the present study. These differences may explain why in the present study we did not detect a net post-asphyxia brain alkalosis as large as the one described in previous work.

3.1.3 | Brain-protecting systems-level mechanisms

To spare the foetal brain which is critically dependent on oxygenation, asphyxia triggers systems-level endogenous protective mechanisms which are usually referred to as the peripheral chemoreflex or the brain-sparing effect. These protective responses involve vasodilation and vasoconstriction which act to maintain perfusion of vital, highly oxygen-dependent organs like the brain, heart and adrenal glands,[13-15,74] thereby reducing the risk of HIE following BA.[40]

While studies monitoring CBF have shed light on adaptive responses acting to maintain brain oxygenation, it is obvious that CBF data do not provide direct information on the level oxygen in brain tissue during asphyxia. For instance, during asphyxia, CBF increases but at the same time there is not only a large decline in O_2 availability but also a large fall in blood pH, which reduces the oxygen carrying capacity of blood because of the Bohr effect.[75-77] Thus, measurements of PO_2 in brain tissue provide direct quantitative information on this key variable, as is the case in the present study. A direct demonstration of the CO_2-dependence of brain oxygenation during asphyxia is that while hypoxia brought about by lowering ambient O_2 to 9% causes a near-instantaneous fall in brain PO_2 to about one tenth of the baseline level, nearly normoxic conditions prevail in the brain parenchyma when the same level of hypoxia is combined with hypercapnia. The brain PO_2 responses to 9% O_2 during intermittent asphyxia appeared as if this brain-sparing effect is augmented by the steps to the low (5%) O_2 levels—a finding similar to what has previously been reported in response to umbilical cord occlusions in a sheep model of BA.[78] However, the increasing trend in brain PO_2 as observed in the peak levels reached by the end of each 9% O_2 period (see Figure 2 bar graph insets) does not differ from that seen during steady 9% O_2 asphyxia, indicating a similar augmentation of the chemoreflex during steady and intermittent asphyxia in the present experimental setting.

Our finding that not only brain PO_2 but also body PO_2 (measured subcutaneously) increased upon hypercapnia does not imply that the two are based on identical mechanisms. Subcutaneous PO_2 increases upon hypercapnia also in humans, but this effect is caused by an increase in cardiac output,[79,80] and not by vasodilation, which is known to be the key mediator of the increase in CBF during hypercapnia.[13]

The results obtained using P6 and P11 rats showed only minor differences between these two developmental stages. Thus, as far as systems-level metabolic and brain-sparing aspects of asphyxia are considered, our model can be used to study perinatal asphyxia at different stages of cortical development, corresponding to preterm to term human babies. Inducing asphyxia via the inhaled gas provides a straightforward method to generate both steady and intermittent asphyxia protocols. These protocols correspond, in rough terms, to two mechanistically different perinatal complications: to acute placental insufficiency and to prolonged periods of uterine contractions respectively.

3.2 | Graded restoration of normocapnia

As discussed above (see Results), the acidosis during experimental asphyxia had two distinct components, respiratory and metabolic, whereof the former is larger in amplitude in both brain and body, and caused by hypercapnia. GRN slowed down the pH recovery after asphyxia by prolonging the duration of respiratory acidosis, and prolonged the overshoot in brain and body PO_2. Whether these effects are beneficial with regard to outcome following asphyxia is not immediately obvious. However, a post-asphyxia seizure-suppressing action of lower brain pH during GRN can be readily assumed (see above, and eg ref. [73]) and this kind of an effect has been directly demonstrated in a parallel study by Ala-Kurikka *et al.*[60]

By definition, hypercapnia causes acidosis without causing any base deficit; and the generation of the base deficit is strictly dependent on metabolic (lactic) acidosis.[81,82] This implies that the lower pH maintained by GRN during recovery from asphyxia does not enhance the base deficit. It may be speculated that since acidosis, no matter if metabolic or respiratory, can limit lactic acid generation,[83] GRN may even assist in the restoration of normal base content.

Spontaneous hypocapnia resulting from hyperventilation is not uncommon in asphyxiated term babies, and hypocapnia occurs often in ventilated – especially preterm – infants. An association between hypocapnia and adverse outcome has been shown in clinical studies on neonates with HIE.[84] A neuroprotective effect of CO_2 against hypoxic-ischaemic injury has been demonstrated in rats[85] and piglets[86] during hypoxia which, in fact, converts the experimental insult from hypoxia to asphyxia. Hypocapnia is particularly injurious to the preterm human brain during the first days of life as it causes brain hypoperfusion and often leads to development of periventricular leukomalacia.[87,88] Mild permissive hypercapnia has been repeatedly suggested as a safe manipulation that can reduce lung injury because of bronchopulmonary dysplasia in ventilated preterm neonates, however, no general recommendations for its optimal use have existed and the levels of hypercapnia with beneficial rather than adverse consequences remain unclear.[89-91] Regarding the current situation with contradicting studies on this issue, it is worth pointing out that the above studies on permissive hypercapnia focus on blood gases in human newborns over a time period of several days or weeks after birth. For instance, in a study on ventilated very low birth weight preterm infants, hypercapnia during the first week of life was found to lead to a progressive loss of cerebral autoregulation with an associated risk of brain injury.[92] In contrast, GRN as studied here, is applied immediately after asphyxia, corresponding to the first hour of extrauterine life after a complicated birth. This is the time when severely asphyxiated newborns often have unstable blood gas levels with episodes of severe hyperoxaemia and severe hypocapnia (arterial PO_2 and $PCO_2 > 200$ mmHg and <20 mmHg, respectively), where these fluctuations *per se* are thought to have a major contribution to brain injury.[93] Given the steep dependence of PO_2 on PCO_2, a strategy to maintaining stability in PO_2 might be based on permissive hypercapnia or on a modification of GRN.

The transient overshoot that was seen in brain and body PO_2 in all our experiments during the first 15 minutes of recovery from asphyxia with RRN is readily accounted for by normal air becoming suddenly available when the endogenous compensatory mechanisms triggered by asphyxia are still acting. In addition to increased CBF, inhibition of mitochondrial respiration and reduced oxygen consumption are likely to be involved.[94-96] The transient PO_2 overshoot is most likely exaggerated in P6 or P11 rats that have acquired fully functional pulmonary gas exchange unlike human newborns, and therefore the amplitude and duration of the PO_2 transient during early recovery should be considered within the framework of the model. Notably, in experiments with RRN, the overshoot was followed by a tendency towards hypoxic PO_2 levels for over an hour in experiments with RRN, whereas elevated PO_2 levels were seen in both brain and body as long as the inhaled gas contained CO_2 in the GRN experiments. These data point to a beneficial action of GRN on brain oxygenation during the onset of pulmonary ventilation.

The brain hyperoxia seen during GRN might raise concerns about its neurotoxic effects. However, unlike with hyperoxic resuscitation[97] the fraction of inspired O_2 is not elevated during GRN, and the PO_2 peak associated with GRN is briefer than what is typically used in neonate animal models of oxygen toxicity.[98] More importantly, hyperoxia occurs in parallel with the GRN-induced hypercapnic acidosis. Therapeutic hypercapnia during reperfusion has been shown to attenuate inflammation and to reduce free radical-mediated injury in an *in vivo* rabbit model of ischaemic lung injury,[99,100] and slowing down the abrupt increase in pH during reoxygenation was found to reduce anoxic injury in perfused rat livers.[11] Based on their own and others' data Halestrap and coworkers conclude that significant superoxide production in the mitochondrial matrix is unlikely during the first minutes of reperfusion in cardiac cells, and that a large increase in intracellular reactive oxygen species occurs only after opening of mitochondrial permeability transition pores,[12,103] which remain inhibited at pH values below 7. Thus, there are reasons to think that the short-term hyperoxia that is linked to the GRN-based hypercapnic acidosis is not detrimental as such.

Taken together, in the present work, the rationale of using GRN as a brain protecting intervention during early recovery from asphyxia is based on augmenting endogenous neuroprotection[40] by making the activity of the brain-sparing mechanisms outlast the asphyxia period. Here, we want to emphasize that using the present GRN protocol is a translational proof-of-concept study, where the amplitudes and durations of the descending CO_2 levels (10% and 5% CO_2) are not intended to be tested as such in the clinic. In fact, we have convincing preliminary evidence[60] that a much milder GRN protocol based on 5% CO_2 leads to a suppression of neocortical seizures following the intermittent asphyxia paradigm in P11 rats.

3.3 | The blood-brain barrier

V_{BBB} is a transendothelial potential difference that prevails between brain tissue and blood. Respiratory pH changes cause large shifts in V_{BBB} that have been measured in invasive recordings in experimental animals and at scalp in human subjects.[48,49] In the present experiments the reference electrodes of the brain and body pH microelectrodes measured tissue potentials, and V_{BBB} was obtained as their difference. V_{BBB} responses to asphyxia were smooth and they appeared to follow primarily the changes in body pH (≈ blood pH) – this was evident during hypoxia when brain pH and body pH shifted in opposite directions. However, there

are no grounds to assume that V_{BBB} depends only on one variable, since it originates as the difference of the apical and basolateral membrane potentials of the BBB forming cells. If a large-scale BBB disruption occurs, it shunts V_{BBB} and a transient shift is seen in the V_{BBB} signal.[14,105] We did not find any indication of robust BBB disruption upon asphyxia in the present experiments. Instead, the present results suggest that BBB remained at least largely if not fully intact.

The fact that V_{BBB} can be easily measured noninvasively[49,105] raises the idea that using one EEG channel dedicated to the monitoring of DC shifts may open up a new window for brain monitoring following BA.

4 | MATERIALS AND METHODS

4.1 | Ethical approval

All experimental procedures were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and the Directive 2010/63/EU, implemented in Finland in Act 497/2013 and Decree 564/2013 on the protection of animals used for scientific or educational purposes. All experiments were approved by the Local Animal Ethics Committee of Helsinki University and the National Animal Ethics Committee in Finland.

4.2 | Animals

Experiments were performed on male Wistar rats on P6 (n = 96) or P11 (n = 43), or on P0 to P2 guinea pigs of either sex (n = 5). Since none of the responses was tested multiple times using the same animal, *n* indicates the number of experiments as well as animals throughout the paper. Rats were obtained immediately before experiments from an in-house animal facility operating under control of the Laboratory Animal Centre of the University of Helsinki (LAC), where rats were housed in cages under 12-hour light/dark cycle and with access to food and water *ad libitum*. Guinea pigs were maintained under similar conditions in a LAC facility from where they were obtained no longer than 30 minutes before they were anaesthetized for surgery.

4.3 | Surgery and preparation for the recordings

Animals were anaesthetized with 4% isoflurane in room air, 1 mg g^{-1} urethane was given by intraperitoneal injection and isoflurane was reduced to 1.5–2% for surgery. After removal of the scalp skin and soft tissue, craniotomies were made for the electrode implantation, including: (1) craniotomies over the hippocampi for the pH (two 0.9 mm holes contralateral to each other) and PO_2 (one 0.9 mm hole) recordings; (2) craniotomy in the occipital bone for the ground wire (placed over the cerebellum). The exposed dura was gently opened using a fine needle to allow electrode implantation. Skin incisions were made on the lower back for the subcutaneous placement of the body pH and PO_2 recordings. At the end of surgery, an additional dose of 0.5 mg g^{-1} urethane was injected and isoflurane removed completely. If animal reacted to tail pinch during sensor placement, an additional dose of urethane (0.5 mg g^{-1}) was injected before the beginning of baseline recording.

After surgery, the animal was placed on a warming pad in the experimental setup, with its head fixed to a stereotactic device. Body temperature was controlled with a rectal probe and BAT-12 thermometer (Physitemp, New Jersey, USA), and the heating was adjusted to maintain body temperature at the typical therapeutic hypothermia level of 33.5-34.0°C during baseline recording in rats to avoid mortality during hypoxia and asphyxia.[39] In the guinea pig experiments, heating settings were kept similar to those used with rats. A piezo movement sensor (PMS20S, Medifactory, Heerlen, The Netherlands) was attached on the lower part of chest with tape to record the respiratory rhythm.

4.4 | pH and PO_2 recording

Commercial H^+-sensitive glass-membrane pH microelectrodes, models pH-25 and pH-500, as well as Clark-type polarographic O_2 microsensors, models OX-10 and OX-N (Unisense A/S, Aarhus, Denmark) were used for pH and PO_2 recordings in brain and body respectively. Tip diameters of the sensors were: 10 μm for brain PO_2, 25 μm for brain pH, 500-750 μm for body pH and PO_2. Glass capillary micropipettes with tips broken to an approximate outer diameter of 20 μm and filled with 0.9% NaCl were used as reference electrodes for pH recording. Brain and body pH signals and their reference-electrode signals were recorded using custom-made electrometer amplifiers and an extracellular field potential amplifier (EXT-02F/2, npi electronic GmbH, Tamm, Germany). Brain and body PO_2 signals were recorded using a PA2000 Picoammeter (Unisense A/S, Aarhus, Denmark). Signals were anti-alias filtered and digitized using Micro1401-3 converter (CED, Cambridge, UK), with sampling frequencies of 100 Hz for breathing and 10 Hz for pH, PO_2 and temperature, and recorded on hard disk with Spike 2 software. The blood-brain barrier potential (V_{BBB}) was measured as the difference between brain and body reference electrodes.

Coordinates for the brain probe implantations were 3-3.5 mm posterior, 3-4 mm lateral, 2.5-3 mm depth from

bregma for the P6 and P11 rats, 5 mm posterior, 6 mm lateral, 3.5 mm depth from bregma in guinea pigs. The reference electrode for brain pH recording was always contralateral to the pH microelectrode, and ipsilateral to the brain PO_2 microsensor in animals with simultaneous brain PO_2 and pH recording. Body pH, PO_2 and reference electrodes were placed subcutaneously, with tips advanced at least 10 mm from the skin incision. Skin incisions were covered with silicone grease to prevent air from accessing the site of recording. In contrast with other probes used in this study, body PO_2 baseline values, unlike the responses to changes in experimental conditions, were slightly influenced by the positioning of the probe, resulting in a wider range of baseline values.

4.5 | Calibration of pH and PO_2 sensors

Calibration of all pH and PO_2 sensors used was done before and after each experiment. pH electrodes were calibrated with their reference electrodes using two solutions containing 150 mmol L^{-1} NaCl and 20 mmol L^{-1} HEPES, pH adjusted to 6.8 and 7.8 with NaOH. The exact pH values of calibration solutions were regularly checked with a standard laboratory pH meter. O_2 sensors were calibrated using standard extracellular solution[24] bubbled for at least 30 minutes with two gas mixtures, one containing 0% O_2 (5% CO_2 in N_2) and the other containing 5 or 9% O_2 and 5-20% CO_2 in N_2. All calibrations were done at room temperature, and a temperature correction was applied during data analysis.

Since pH calibrations were done at room temperature, the temperature sensitivity of the differential pH recording was found out experimentally, not forgetting the temperature dependence of the pKa of HEPES used in the calibration solutions. Based on the results, a correction of −0.09 pH units was applied to tissue pH values. Differences in observed brain or body pH baseline values between individuals in a cohort reflect true differences in pH as well as random sources of error characteristic of the method. The latter is likely to dominate, and therefore baselines of individual recordings were offset to the corresponding means.

The signal of polarographic O_2 microsensors and the solubility of gaseous O_2 are temperature dependent. Thus, in order to quantify the data in terms of partial pressure, we analysed the overall effect of these temperature dependencies using solutions that were vigorously bubbled for at least half an hour at room temperature (20-21°C) and at 34°C with gas mixtures containing 0%, 5% or 9% O_2, and found a temperature dependence of ~1% per °C. Brain and body PO_2 sensor data were corrected accordingly during data analysis. At very low tissue PO_2 values (sensor current close to 0 pA) that typically occurred during 5% hypoxia or asphyxia, the PO_2 trace was still fluctuating or it was steady, showing no fluctuation for several minutes. In experiments where the latter condition occurred, the non-fluctuating level was taken as "true zero", and the PO_2 trace was offset accordingly. The average offsets of this kind were 1.5 mmHg and 2.5 mmHg for brain and body PO_2, respectively, and applied to traces where a true zero condition was not seen during the experiment.

4.6 | Experimental protocols

After initial stabilization of the recorded signals, a 30 minutes baseline was recorded from each animal breathing humidified room air applied via a small-rodent facemask (model OC-MFM for rats, OC-LFM for guinea pigs, World Precision Instruments), at a flow rate of approximately 1200 ml min^{-1}. The humidified experimental gas mixtures (AGA [Linde Group], Finland) were applied at the same rate, and were as follows: 5% or 9% O_2 and 20% CO_2 in N_2 (asphyxia); 5% or 9% O_2 in N_2 (hypoxia); 5%, 10% or 20% CO_2 and 20-21% O_2 in N_2 (hypercapnia; the first two also for GRN). For clarity, the timing of gas applications is given in the Figures using schematic traces.

4.7 | Lactate measurement

Blood lactate was measured with a GEM Premier 4000 blood gas analyser (Instrumentation Laboratory). P6 rats were exposed to 5% asphyxia for 15 minutes, immediately after which blood was collected and analysed as described before.[47]

4.8 | Data processing and statistics

Data were processed using custom-made scripts in Matlab (MathWorks Inc.), and with Excel (Microsoft) and SigmaPlot 14 (Systat Software, Inc.).

All numerical data are given as mean ± SEM (including pH that is a logarithmic measure of proton activity; see Supporting Information). Differences between mean values were assessed using unpaired or paired *t*-tests, and if not specified the test was unpaired. $P < .05$ was considered statistically significant. We are aware of the limitations of the *t*-test when sample sizes are small, and we give *P* values as suggestive information only. The reader might appreciate the low variability in the primary data, which is evident in the Results.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

KK and JV conceived the study and designed the experiments with AP and MP. The data were collected by AP and analysed by AP and JV. All authors were involved in data interpretation and writing the manuscript, and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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